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Antioxidant Property of Aqua-Acupuncture Solution from Circium japonicum

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대계 약침액의 항산화 효능

이 정 주, 문 진 영

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한글 초록

대계 (大薊)는 전통 한의학에서 약용 물질로 널리 쓰이고 있으므로, 대계의 약침을 조제하여 항산화적 활성을 검증해 보았다. 실험의 재료로 쓰인 대계는 동국대학교 한의과대학 부속 한방 병원에서 구입하여 사용하였고 (경상북도 경주), 이를 약침액으로 조제한 후 동결 건조 방법으로 5.1 %의 분말을 회수 하였다. 대계 약침액의 항산화 능력을 측정하기 위하여 DPPH 자유기의 소거능은 Gyamfi et al. 의 방법을 따랐고, superoxide radicals (O_2^-·) 소거능은 Gotoh et al.의 방법을 일부 수정하여 사용하였다. Hydroxyl radicals의 직접적인 소거능과 iron ions 사이의 관계는 deoxyribose assay를 통하여 검증하였다. 이상의 실험 결과에서 1) Non-site-specific scavenging assay (hydroxyl radicals, OH) 및 2) Site-specific scavenging assay (chelate iron ions) 그리고 3) 전산화 효과에서 모두 대계 약침액은 농도 의존적인 저해 효과를 보였으며, 특히 pro-oxidant 실험에서는 산화를 촉진하지 않는 것으로 나타났다. 끝으로 hydorxyl radical에 의하여 매개되는 DNA의 분절 효과를 적절하게 방어하는 것을 관찰할 수 있었다.

따라서 대계 약침액은 산화적 스트레스를 직접적으로 방어 하고, 다양한 산화적 손상에 의한 병증을 예방할 것으로 사료된다.

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Ⅰ. Introduction

Reactive oxygen spices (ROS) such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion ($O_2^-\cdot$), and hydroxyl (·OH) radical are often generated as byproducts of biological reaction or from exogenous factor[1]. The involvement of these species in the pathogenesis of a large number of diseases including rheumatoid arthritis, atherosclerosis, skin aging, nephrites, reperfusion injury, asthma, diabetes mellitus, and carcinogenesis are well documented[2-6]. A potent scavenger of these species may serve as a possible preventive intervention for free radical-mediated diseases[7]. In recent years, there has been a global trend toward the use of natural phytochemicals present in natural resources, such as fruit, vegetables, oilseeds, and herbs, as antioxidants and functional foods[8-10]. In traditional Korea and Chinese medicine, aqua-acupuncture solution are used for various diseases. The Circium japonicum is founded wild in Korea, and is used as a medicinal herb in Korea[11-12]. However, very little information is currently available on the active constituents present in CJAS the are responsible for its antioxidant activity. Futhermore, the mechanism of the antioxidant actions of CJAS has yet to be determined. Hence, the purposes of this work were to determine the antioxidant property of CJAS and to preliminarily define the active components and mechanism of its antioxidant action.

Ⅱ. Materials and Methods

1. Plastics and Chemicals

Unless otherwise specified, all the plastics were from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ) and 1,1-diphenyl-2-picrylhydrazyl (DPPH), L-ascorbic acid (vitamin C), tris aminomethane (trizma base), hydrochloride, EDTA, hypoxanthine, xanthine oxidase, NaOH, nitro blue tetrazolium (NBT), deoxyribose, hydrogen peroxide (H_2O_2), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), ferric chloride ($FeCl_3$) were purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A). Agarose, ethidium bromide (EtBr), and 6X orange-blue dye were obtained from Promega (Promege, Madison, WI). All other chemicals used analytical grade and purchased from either Merck (Merck KGaA, Germany) or Junsei (Junsei Chemical Co., Ltd. Japan).

2. Preparation of CJAS Extracts

Circium japonicum was obtained from a Dongguk Korean Medicine Hospital (Kyungju, Kyungbuk). A 60 g sample of crude plant was cut into small pieces and then CJAS added 500 ㎖ of water and the suspension boiled for 3 h at 80 ℃. The crude preparation was the boiled with absolute ethanol (final concentration 75%, 85% and 90%) for 3 days. The resulting aqua-acupuncture solution was subsequently filtered through filter paper (Whatman No.#1) and centrifuged at 4,000 rpm for 10 min. The collected supernatant was freeze-dry to give a final weight 3.04 g (yield 5.1%)

for the dry powder of aqua-acupuncture solution. A voucher specimen has been deposited in the herbarium of the Acupoint & Meridian Laboratory.

3. Scavenging Activity onto DPPH Radicals

Scavenging activity on DPPH free radicals by the CJAS was assessed according to the method followed by Gyamfi et al[13]. Briefly, 50 ㎕ of the CJAS containing varied amounts of powered extract (0.1 to 8 ㎎/㎖ distilled water, respectively, in each reaction) was mixed with 1 ㎖ of 0.1 mM DPPH (dissolved absolute ethanol) solution and 450 ㎕ of 50 mM Tris-HCl buffer (pH 7.4). After the solution incubated for 30 min at room temperature, reduction of DPPH free radicals was measured by reading the absorbance at 517 nm. In this experiment, dose dependent effects of CJAS as well as L-ascorbic acid on the reduction of the DPPH radicals was also determined. The inhibition percent was calculated from the following equation :

% inhibition = [(Abs_{517} of control - Abs_{517} of sample)/Abs_{517} of control] × 100

4. Scavenging Activity onto Superoxide Anions

Scavenging activity on superoxide radicals ($O_2^{-}\cdot$) was assessed by the method described by Gotoh et al[14]. with slight modification. Briefly, different concentrations of the CJAS were added to the reaction solution containing 100 ㎕ of 30 mM EDTA (pH 7.4), 10 ㎕ of 30 mM hypoxanthine in 50 mM NaOH, and 100 ㎕ of 3 mM nitro blue tetrazolium (NBT). After the solution was preincubated at room temperature for 3 min, 100 ㎕ of 0.5 U/㎖ xanthine oxidase was added to the mixture and the volume was brought up to 3 ㎖ with 50 mM phosphate buffer (pH 7.4). After the solution was incubated at room temperature for 20 min, absorbance was measured at 560 nm.

5. Deoxyribose Assay

Deoxyribose assay to determine the rate constant for the reactions between either antioxidants and hydroxyl radicals or antioxidants and iron ions was conducted as described by Halliwell et al[15]. with slight modification.

(1) Non-site-specific scavenging assay (hydroxyl radicals directly, · OH)

50 ㎕ of the CJAS containing varied amounts of powered extract was mixed with 1 ㎖ reaction buffer (0.1 mM $FeCl_2$, 0.1 mM EDTA, 1.5 mM H_2O_2, 2.5 mM deoxyribose, and 0.1 mM L-ascorbic acid, pH 7.4) and incubated for 1 hr at 37 ℃. After the solution were added to 1 ㎖ 0.5 % TBA in 0.025 M NaOH and 1 ㎖ of 2.8% TCA and heated for 30 min at 80 ℃. Finally, the mixture was cooled on ice and absorbance was measured at 532 nm using a spectrophotometer (UV2120+, MECASIS, Korea).

(2) Site-specific scavenging assay (chelate iron ions)

The ability of CJAS to chelate iron ions and interfere with hydroxyl radicals generation, was measured using the same reaction

buffer without EDTA.

(3) Pro-oxidant effect of the CJAS on iron dependent hydroxyl radical generation

The ability of the CJAS to reduce the Fe^{3+}-EDTA complex to Fe^{2+}-EDTA complex, acting as pro-oxidant, was tested using the non-site-specific assay mixture without the addition of ascorbic acid.

6. DNA Nicking Assay

A DNA nicking assay was performed using supercoiled pBR322 plasmid DNA prepared from DH5α using Plasmid Miniprep Kit (JBI and Welgene, Deagu, Korea)[16]. 0.5 μg of plasmid DNA was added to Fenton's reagents (30 mM H_2O_2, 0.05 mM ascorbic acid, and 0.08 mM $FeCl_3$) containing different concentration of the CJAS, and the final volume of the mixture was brought up to 20 $\mu\ell$. The mixture was then incubated for 30 min at 37 ℃ and the DNA was analyzed on a 1.2 % agarose gel followed by ethidium bromide staining.

7. Statistical Analysis

Experimental results were mean±S.D. tree parallel measurements. Analysis of variance was performed by Sigma Plot procedure (ver. 6.1 for Windows). Significant differences between means were determined by Student's t-test. p values<0.05 were regarded as significant.

Ⅲ. Results

1. Scavenging Effect of CJAS on DPPH Radicals

The free radical scavenging activity of antioxidants is considered to be due to their hydrogen-donation ability, we used a method based on the reduction of DPPH a stable free radical, to evaluate the antioxidant activity of various CJAS. DPPH has been widely used to test the free radical scavenging activity of various samples. As shown in Figure 1, the CJAS significantly inhibited the activity of DPPH radicals in a dose-depentent manner. But, almost low level inhibition of the anti-DPPH radical activity was observed when high-concentration of CJAS was used.

2. Scavenging Effect of CJAS on Superoxide Anions

Next, we test the scavenging effect of CJAS on superoxide anions by monitoring the reduction of NBT induced by superoxide anions produced by the xanthine oxidase-mediated degradation of hypoxanthine. Superoxide anion radicals are prduced endogenously by flavoenzymes, xanthine oxidase, which converts hypoxanthine to xanthine and subsequently to uric acid in ischemia-reper-fusion. Superoxide is generated in vivo by several oxidative enzyme, including xanthine oxidase. Figure 2 shows that CJAS inhibited NBT reduction very efficiently. CJAS had strong superoxide radicals scavenging activity and than activity was similar to that BHA (data not shown).

3. Hydroxyl Radical Scavenging: the Deoxyribose assay

We further investigated the mechanisms involved in CJAS -mediated antioxidant activity, and particularly to determine whether the CJAS extract decreases hydroxyl radical generation by chelating metal ions or by directly scavenging hydroxyl radicals, the effect of CJAS on hydroxyl radicals generated by Fe^{3+} ions was measured by determining the degree of deoxyribose degradation, an indicator of TBA-malonaldehyde adduct formation. As shown in Figure 3, concentration-dependent inhibition of hydroxyl radical-induced deoxyribose degradation was observed in the site-specific and non-site-specific assays. In contrast, when the ability of CJAS to reduce the Fe^{3+}-EDTA complex was tested to examine its pro-oxidant activity, there was completely pro-oxidant activity and then pro-oxidant effect was not observed all the concentration (Table 1).

Table 1. Pro-Oxidant Effect of CJAS on Iron-Dependent Hydroxyl Radical Generation

CJAS (mg/mℓ)	Optical density (Abs 532 nm)	% stimulation
control	1.943	-
0.25	1.782	0 %
0.5	1.737	0 %
1	1.795	0 %
2	1.960	1 %
4	1.789	0 %
8	1.804	0 %

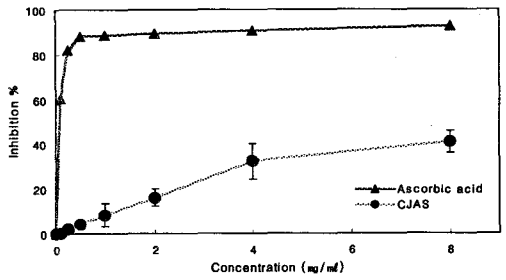

Figure 1. Free radical scavenging activity of CJAS measured using the DPPH assay. The direct scavenging activities of CJAS and ascorbic acid on DPPH radicals is expressed as the % inhibition. The concentrations tested ranged from 0.1 to 8 mg/mℓ. The results are the means of three separate experiments.

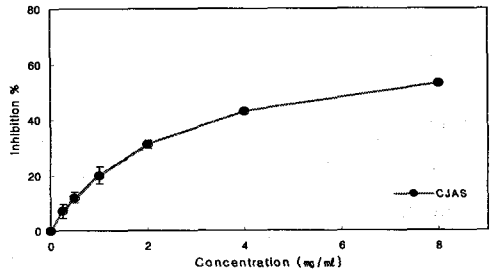

Figure 2. Inhibitory effects of CJAS on NBT reduction. The inhibitory effects of CJAS was tested by monitoring NBT reduction caused by superoxide anions using the hypoxanthine-xanthine oxidase system, as described in the Materials and Metheds section. The results are expressed as the mean values of triplicate experiments.

4. Scavenging Effect of CJAS on Fe3+-Dependent DNA Nicking

To measure the scavenging effect of CJAS on Fe^{3+}-dependent hydroxyl radicals, we investigated whether the sample reduced Fe^{3+}-dependent DNA nicking (Figure 4, B).

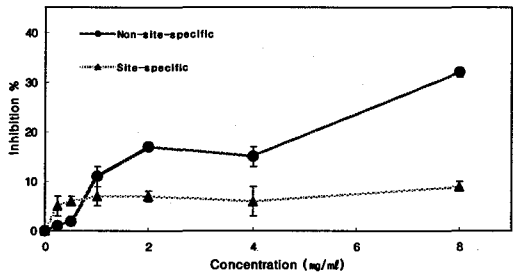

Figure 3. Inhibitory effects of CJAS on hydroxyl radical-mediated deoxyribose degradation. Hydroxyl radicals were generated by Fenton's reaction using a deoxyribose assay system, and the non-site-specific (●) and site-specific (▲) scavenging activities of hydroxyl radicals by CJAS are expressed as the % inhibition. The concentration of CJAS tested ranged from 0.1 to 8 ㎎/㎖. The results are the means of three separate experiments.

When pBR322 plasmid DNA was dissolved in the reaction mixture, a time dependent increase in the formation (Figure 4, A) of single-stranded nicked DNA and of double-stranded nicked DNA and linear DNA was observed. However, the addition of 1 to 4 ㎎/㎖ of CJAS to the nicking reaction mixture increased super-coiled DNA formation. Consequently, the treatment caused Fe^{3+}-mediated double-stranded DNA formation to disappear and reduced single-stranded DNA formation.

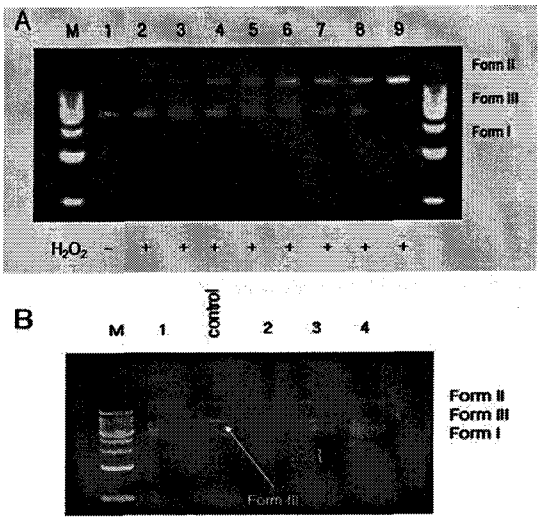

Figure 4. Inhibitory effect of CJAS on DNA nicking caused by hydroxyl radicals. The DNA nicking reaction were initiated by the addition of 0.5 ㎍ of pBR322 plasmid DNA.
(A) Lanes 2 through 9 represent the results from the mixture incubated for 0, 5, 10, 20, 30, 45, 50 and 60 min, respectively. Lanes M and 1 show the size maker and native plasmid DNA, respectively.
(B) Lanes M and 1 show the size maker and native plasmid DNA, respectively. Lanes 2, 3 and 4 show the CJAS treated range from 0.1 to 4 ㎎/㎖.
Form I : Supercoiled DNA
Form II : Single strand nicking from
Form III : Double strand nicking form

Ⅳ. Discussion

The indirect evidence of the scavenging activity of CJAS on Fe^{3+}-dependent hydroxyl-radicals generation was further confirmed using a direct approach with DPPH radicals, a stable radical used to evaluate the antioxidant activity of plant and medicinal herb[17-18]. In this assay, the CJAS exhibited weakly DPPH radical scavenging activity and the activity was lower than ascorbic acid (Figure 1). But we suggesting that CJAS is a natural antioxidant. Superoxide anions are the most common free radicals in vivo and are generated in a vatiety of biological systems, either by auto-oxidation processes or by enzymes. The concentration of superoxide anions increase under

conditions of oxygenative stress and related situations[14,19-20]. Moreover, superoxide anions produce other kinds of cell-damaging free radicals and oxidizing agents[21]. Therefore, we used the NBT assay system to test whether CJAS scavenge superoxide anions. In the system, xanthine oxidase, which is one of the main enzymatic sources of ROS in vivo, generated superoxide radicals, which consequently reduced NBT to yield blue formazan. The active inhibition of the NBT reduction induced by hypoxanthine-xanthine oxidase by CJAS (Figure 2) suggests that CJAS is a potential scavenger of superoxide anions, as well as hydroxyl radicals. Next, we used a deoxyribose assay system to confirm the antioxidant activity of CJAS, and found that the its inhibited hydroxyl radical-induced deoxyribose degradation in both non-site-specific and site-specific assays (Figure 3). In particular, the CJAS inhibited deoxyribose degradation by hydroxyl radicals directly rather than by chelating iron ions. The possible pro-oxidant activity of CJAS was also assessed using deoxyribose assays, because it might become biologically available to catalyze a free radical reaction at sites of tissue injury, especially in advanced atherosclerotic lesions and chronic inflammation[22]. However, its ability to reduce the Fe^{3+}-EDTA complex, which represents its ability to stimulate hydroxyl radical generation, was not observed low to high concentration of the CJAS were added (Table 1). These results suggest that the pro-oxidant activity of CJAS is readily overwhelmed by the antioxidant potential of the extract. Finally, we initially used an Fe^{3+}-dependent system to test the scavenging activity of CJAS on radicals generated by iron, because hydroxyl radicals are known to be the most reactive of all the reduced forms of dioxygen and are thought to initiate cell damage in vivo[23]. The results of DNA experiments showed that the CJAS was an active scavenger of hydroxyl radicals, such that DNA nicking was siginificantly prevented by the presence of CJAS (Figure 4).

In summary, it is well understood that the generation of ROS beyond the capacity of a biological system to eliminate them gives rise to oxidative stress. This stress may play a role in several diseases, such heart disease, degenerative neuronal disease, and cancers[20,24-26]. Our study demonstrated that CJAS has antioxidant activities. Futhermore, many biochemical and clinical studies suggest that natural and synthetic antioxidant compounds are helpful in treating diseases mediated by oxidative stresses.

References

1. Cerutti PA. Oxidant stress and carcinogenesis. Eur J Clin Invest. 1991 ; 21 : 1-11.
2. Stadtman ER, Oliver CN. Metal ion-catalyzed oxidation of protein, physiological consequences. J Biol Chem. 1991 ; 266 : 2005-08.
3. Cerutti PA. Oxy-radicals and cancer. Lancet. 1994 ; 344 : 862-3.
4. Dandona P, Thusu K, Cook S, Snyder B, Makowski J, Amstrong D, Nicotera T.

Oxidative damage to DNA in diabetes mellitus. Lancet. 1996 ; 347 : 444-5.
5. Freig DT, Reid TM, Loeb LA. Reactive oxygen species in tumorigenesis. Cancer Research. 1994 ; 54 : 1890-4.
6. Stadtman ER. Metal ion-catalyzed oxidation of proteins: biochemical mechanisms and biological consequences. Free Rad Biol Med. 1990 ; 9 : 315-25.
7. Ames BN, Gold IS, Willer WC. The causes and prevention of cancer. Proc Natl Acad Sci. 1995 ; 92 : 5258-65.
8. Wang H, Cao G, Prior RL. Oxygen radical absorbing capacity of anthrocyanins. J Agric Food Chem. 1997 ; 45 : 304-9.
9. Kitts DD, Wijewickreme AN, Hu C. Antioxidant properties of a North American ginseng extract. Mol Cell Biochem. 2000 ; 203 : 1-10.
10. Farr DR. Functional foods. Cancer Lett. 1997 ; 114 : 59-63.
11. Lee JJ, Moon JY. Effects of Circii Herba aqua-acupuncture (BL18, CV12) on zcute oxidative liver injury. Kor J Mer & Acu. 2003 ; 20(4) : 41-52.
12. 中華人民共和國衛生部藥典委員會. 中華人民共和國藥典會. 北京 : 人民衛生出版社. 1985 : 17.
13. Gyamfi MA, Yonamine M, Aniya Y. Free-radical scavenging action of medicinal herbs from Ghana. Gen Pharmacol. 1999 ; 32 : 661-7.
14. Gotoh N, Niki E. Rates of interactions of superoxide with vitamin E, vitamin C and related compounds as measured by chemiluminescence. Biochim Biophys Acta. 1992 ; 1115 : 201-7.
15. Halliwell B, Gutteridge JMC, Aruoma OI. The deoxyribose method: simple "test-tube" assay for determination of rate constants for reaction of hydroxyl radicals. Anal Biochem. 1987 ; 165 : 215-9.
16. Lee JC, Kim HR, Kim J, Jang YS. Antioxidant property of an ethanol extract of the stem of Opuntia ficus-indica var. Saboten. J Agric Food Chem. 2002 ; 50 : 6490-6.
17. Hu C, Kitts DD. Studies on the antioxidant activity of Echinacea root extract. J Agric Food Chem. 2000 ; 48 : 1466-72.
18. Chang ST, Wu JH, Wang SY, Kang PL, Yang NS, Shyur LF. Antioxidant activity of extract from Acacia confusa bark and heartwood. J Agric Food Chem. 2001 ; 49 : 3420-4.
19. Mates JM, Sanchez-Jimenez FM. Role of reactive oxygen species in apoptosis: Implications for cancer therapy. Int J Biochem Cell Biol. 2000 ; 32 : 157-70.
20. von Harsdorf R, Li PF, Dietz R. Signaling pathway in reactive oxygen species-induced cardiomyocyocyte apoptosis. Circulation. 1999 ; 99 : 2934-41.
21. Liu F, Ng TB. Antioxidative and free radical scavenging activities of selected medicinal herbs. Life Sci. 2000 ; 66 : 725-35.
22. Aruoma OI, Spencer JPE, Warren D, Jenner P, Butler J, Halliwell B. Characterization of food antioxidants, illustrated using commercial garlic and ginger preparations. Food Chem. 1997 ; 60 : 149-56.
23. Roller-Labelle E, Grange MJ, Elbim C,

Marquetty C, Gougerot-Pocidalo MA, Pasquier C. Hydroxyl radical as a potential intracellular mediator of polymorphonuclear neutrophil apoptosis. Free Radical Biol Med. 1998 ; 24 : 563-72.

24. van Poppel G, van den Berg H. Vitamins and cancer. Cancer Lett. 1997 ; 114 : 195-202.

25. Adams JD, Odunze IN. Oxygen free radicals and Parkinson disease. Free radical Biol Med. 1991 ; 10 : 161-9.

26. Hertog MGL, Feskens EJM, Hollman PCH, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. Lancet. 1993 ; 342 : 1007-11.

Abstract

Objectives : Circium japonicum is a pharmacologically active used in traditional Korean medicine. An aqua-acupuncture solution of the Circium japonicum was assessed to determine the mechanism of its antioxidant activity.

Materials : Circium japonicum was obtained from a Dongguk Korean Medicine Hospital (Kyung-ju, Kyungbuk). The freeze-dry powder was collected (yield 5.1%) for the aqua-acupuncture solution. Scavenging activity on DPPH free radicals by the Circium japonicum aqua-acupuncture solution (CJAS) was assessed according to the method followed by Gyamfi et al.. and then scavenging activity on superoxide radicals ($O_2^{-}\cdot$) was assessed by the method described by Gotoh et al. with slight modification. Deoxyribose assay to determine the rate constant for the reactions between either antioxidants and hydroxyl radicals or antioxidants and iron ions. We tested by; (1) Non-site-specific scavenging assay (hydroxyl radicals, ·OH), (2) Site-specific scavenging assay (chelate iron ions), and (3) Pro-oxidant effect of the CJAS on iron dependent hydroxyl radical generation. Finally, we determined hydorxyl radical-mediated DNA nicking formation.

Conclusion : Our study demonstrated that CJAS has antioxidant activities and we investigated the potential effectiveness of CJAS in preventing oxidative stress-mediated disease further.

Key words : aqua-acupuncture solution, antioxidant, oxidative stress, hydroxyl radicals, DNA nick formation